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Piper nigrum extract attenuates food allergy by decreasing Th2 cell response and regulating the Th17/Treg balance

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Piper nigrum is extensively utilized because of its antioxidation, antiallergic, antitumor, antiinflammatory, antidiarrhea, and gastrointestinal protection. We attempted to indicate whether the *Piper nigrum* extract (PNE) could alleviate ovalbumin (OVA)-induced food allergy, and to explore its potential mechanism. An OVA-induced food allergy mouse model was established, and different concentrations of PNE were administered. Symptoms of food allergy, levels of immunoglobulin E (IgE), mucosal mast cell protease-1 (mMCP-1), and intestine pathological changes were assessed. Additionally, the expressions of T helper (Th) 2, Th17 and regulatory T (Treg)-associated cytokines and the proportion of Th17 and Treg cells in CD4⁺ T cells were measured. We found PNE attenuated symptoms of food allergy and decreased the levels of IgE and mMCP-1. In PNE group, the infiltration degree of inflammatory cells was ameliorated and the villi of small intestine were more complete. Moreover, the expressions of Th2 and Th17 cell-associated cytokines were down-regulated by PNE pretreatment, while the levels of Treg cell-associated cytokines were up-regulated. PNE decreased the number of Th17 cells, while increased the Tregs cells. PNE treatment dose-dependently improved the Th17/Treg balance. PNE plays a protective role in OVA-induced food allergy through inhibiting Th2 cell response and regulating the Th17/Treg balance.

KEYWORDS

food allergy, *Piper nigrum* extract, Th17, Th2, Treg

1 | INTRODUCTION

Food allergy is a pathologically and potentially fatal immune response caused by harmless food protein antigens, in which its typical symptoms include abdominalgia, diarrhea, vomiting, nettle rash, angioedema, and even anaphylactic shock (Jones & Burks, 2017). The incidence rate of food allergy is rapidly increasing and it is currently taken as a main public health concern into account (Prescott et al., 2013). Activated mast cells, eosinophils, neutrophils, effector T helper (Th) cells, as well as released inflammatory cytokines, and chemokines are all closely associated with food allergy (Francis

et al., 2017; Reber et al., 2013; Sampson et al., 2018). When food allergens pass through the epithelial barrier, naïve T cells differentiate into Th2 cells and release interleukin (IL)-4, IL-5, and IL-13, which can promote B cells to release immunoglobulins, especially immunoglobulin E (IgE) (Yu, Freeland, & Nadeau, 2016). When antigens cross-link with IgE and bind to FcεRI on the surface of mast cells, they may stimulate degranulation of mast cells, causing the development of allergy symptoms (Albert et al., 2019). In addition, when Th2 response is enhanced, it can inhibit the response of regulatory T (Treg) cells to inflammation (Tordesillas, Berin, & Sampson, 2017). Gupta, Gupta, and Dwivedi (2017) found that Th17-associated IL-17A was indispensable in the process of food allergy. Besides, Treg cells effectively prevent development of food allergy by suppressing activation of Th2 and

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Th17 cells, in addition to degranulation of mast cells (Georg et al., 2008). Recent researches pointed out that the Th17/Treg balance is associated with allergic diseases (Wei et al., 2015; Xu, Li, Zhang, Zhao, & Zhang, 2017).

Piper nigrum, also called black pepper, is widely utilized because of its antioxidation, antiallergic, antitumor, antiinflammatory, and antidiarrhea activities, as well as gastrointestinal protection (Takoori et al., 2019). Black pepper contains various bioactive components, for example, piperine, which is considered as the main component and exert the key pharmacological actions (Meghwal & Goswami, 2013). Piperine has antiinflammatory effects, which can decrease secretion of proinflammatory factors and promote secretion of antiinflammatory factors in mice (Zhai et al., 2016). A previous study demonstrated that piperine is known for its remarkable immunomodulatory actions, which can affect the response of T cells to inflammation by reducing the expressions of IL-4 and IL-17A (Doucette, Rodgers, Liwski, & Hoskin, 2015). Piperine also has been reported to have antioxidant, antidiarrhea, and antitumor effects (Salehi et al., 2019; Srinivasan, 2009). Recently, the role of black pepper in improving allergic response has attracted scholars' attention. Piperine improves asthma by inhibiting Th2 immune reaction and activation of mast cells (S. H. Kim & Lee, 2009). *Piper nigrum* extract (PNE) can alleviate allergic rhinitis by suppressing the secretion of Th17-associated IL-17A and Th2-associated IL-4, IL-5, and IL-13 (Bui et al., 2019). Nevertheless, whether PNE can improve food allergy has not been studied. In the present research, an ovalbumin (OVA)-induced food allergy mouse model was established, and different concentrations of PNE were administrated to explore whether PNE has a protective effect on food allergy, and its possible mechanism.

2 | MATERIALS AND METHODS

2.1 | Animals

Female BALB/c mice (5-week-old, 18–20 g) were obtained from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China; SYXK-2018-0017). Before the experiment, mice were kept in our animal laboratory for 1 week, at the temperature of $23 \pm 2^\circ\text{C}$, with relative humidity of 50–70%, and under a 12:12-hr light: dark cycle. According to the random number table method, 30 BALB/C mice were randomly divided into five groups: the control group, the OVA group, the PNE 50 mg/kg, 100 mg/kg, and 200 mg/kg group. All animal experiments were approved by the Ethics Committee of Wenzhou Medical University (Wenzhou, China; Approval No. wydw2018-0008).

2.2 | Preparation of PNE

PNE was obtained from Xinyang Mufan Biotechnology Co., Ltd. (Shanghai, China). According to the manufacturer's instruction, the

extraction ratio of PNE is 1 g extracted from 10 g black pepper (1:10). The medicinal materials (raw black pepper) was purchased from a rural market in Xinyang City (China), and extracted by 70% ethanol under heating condition repeatedly. After that, the extracted ethanol was evaporated and dried in vacuum to obtain a PNE powder, so ethanol has no effect on the follow-up test (Bui et al., 2019, 2020). Finally, PNE powders were kept at 4°C in the Xinyang Mufan Biotechnology Institute. The evidence of chemical characterization of PNE and HPLC of piperine in PNE analysis report were attached in Data S2.

2.3 | Food allergy model

Mice were allocated to five groups ($n = 6$), including control group (saline sensitization/challenge), OVA group (OVA sensitization/challenge), PNE-50 group (OVA + 50 mg/kg PNE), PNE-100 group (OVA + 100 mg/kg PNE), and PNE-200 group (OVA + 200 mg/kg PNE). There are many allergens that can cause food allergy, but egg is one of the most common allergens. We have consulted the relevant literature and established the OVA (ovalbumin)-induced food allergy mice model (Honjo et al., 2017; J. H. Kim et al., 2016; Yamaki & Yoshino, 2012). Briefly, 50 μg OVA (Sigma-Aldrich, St. Louis, USA) and 2 mg aluminum hydroxide and magnesium hydroxide were dissolved in 200 μl normal saline, and intraperitoneally injected into mice for sensitization on the 0th and 14th days. OVA group and PNE-based groups were challenged intragastrically with 50 mg OVA in 200 μl normal saline every 2 days from day 28 to day 38 after sensitization. To investigate the preventive effect, the PNE-based groups were orally administration 200 μl PNE solution at fixed time every day. The drug's oral administration on the day before challenge was carried out at 24 hr before doing the challenge on the next day. The duration of administration on the day of challenge was chosen to be 1 hr before OVA challenge, so as to achieve a better protective effect (Bae et al., 2016; J. H. Kim et al., 2016; Yamaki & Yoshino, 2012). The control and OVA groups were treated with saline (Figure 1A).

2.4 | Evaluation of allergy symptoms

We evaluated diarrhea, anaphylaxis, and rectal temperature at 1 hr after challenging with OVA as indicators of food allergy symptoms, so as to prove that the food allergy model was established successfully (Bae et al., 2016; Honjo et al., 2017; Lee et al., 2018). Scoring of diarrhea was performed in form of a 0–3-point system as follows: 0-point, normal stool; 1-point, a wet and shapeless stool; 2-point, an extremely wet and shapeless stool; and 3-point, an extremely wet stool with serious perianal staining of the coat. Scoring of anaphylactic responses was undertaken in form of a 0–4-point system: 0-point, no symptom; 1-point, decreased activity, quivering of limbs; 2-point, no consciousness and activity after irritating; 3-point, convulsion; and 4-point, death. We measured rectal temperature every 20 min for 1 hr after the sixth OVA challenge.

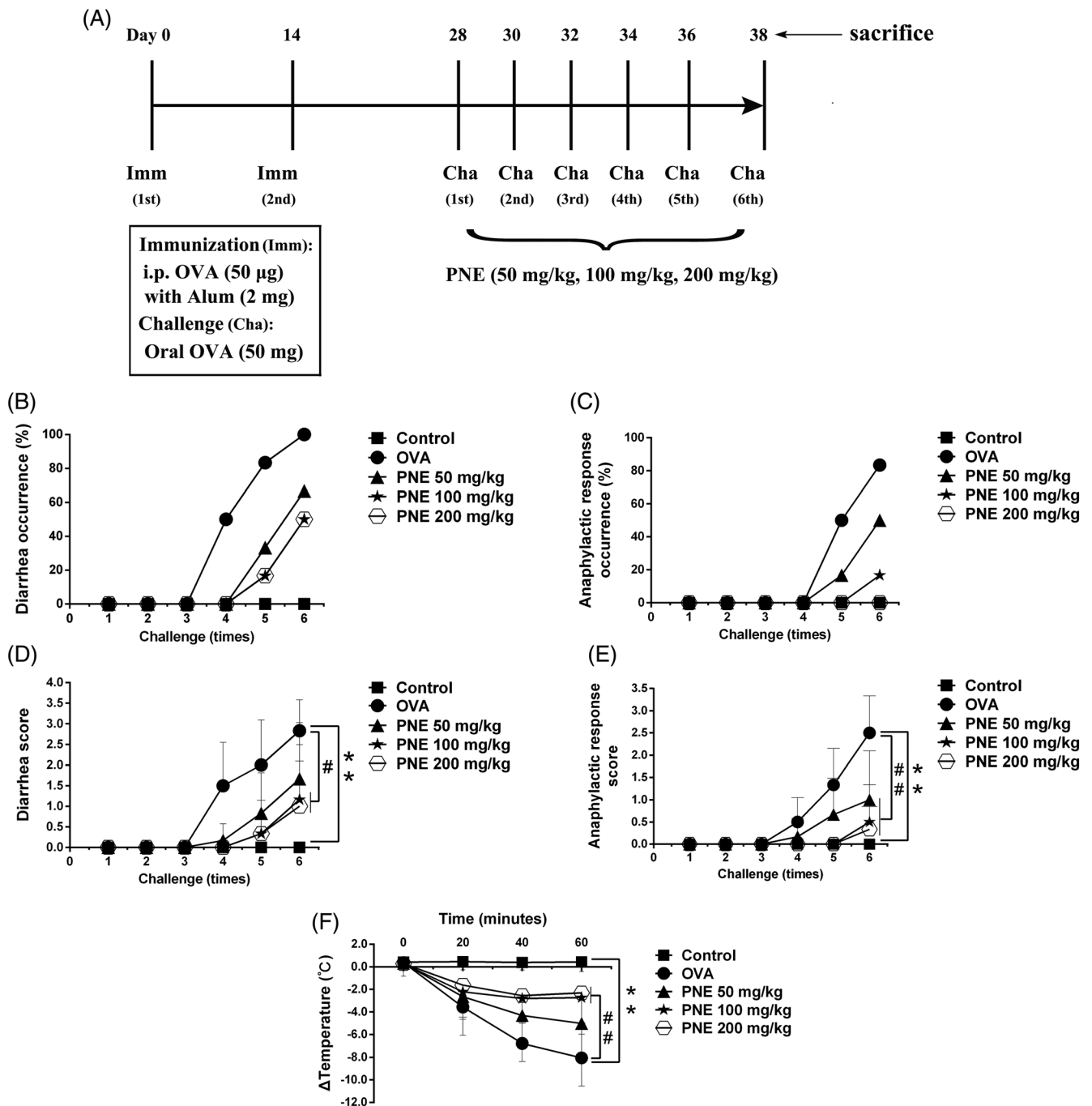


FIGURE 1 PNE attenuated allergy symptoms. (A) Food allergy model. Mice were assigned to control group, OVA group, PNE-50 group, PNE-100 group, and PNE-200 group ($n = 6$). In order to induce allergic reaction, 50 μ g OVA intraperitoneal injection on 0th and 14th days. From the 28th day, the mice were challenged with 50 mg OVA every 2 days for six times. From day 28 to day 38, various concentrations of PNE (50, 100, and 200 mg/kg) were administered every day. Diarrhea, anaphylaxis, and rectal temperature were evaluated for 1 hr after challenge with OVA. (B) The incidence of diarrhea (%; score ≥ 2); (C) the incidence of anaphylaxis (%; score ≥ 2); (D) Diarrhea score; and (E) anaphylaxis score were assessed. (F) Rectal temperature was measured every 20 min for 1 hr after the sixth challenge. All experiments were run in triplicate. Data were presented as mean $\pm$ SD ($n = 6$). ** $p < .01$ compared with control group. # $p < .05$; ## $p < .01$ compared with OVA group

2.5 | Histological examination

The jejunum tissues were fixed with 10% formalin solution and embedded into paraffin. Next, they were cut into 5–6 mm thick sections and stained with hematoxylin and eosin (H&E). The

inflammatory cell infiltration was observed by a light microscope (Nikon, Tokyo, Japan). The intestinal tissue was fixed with electron microscope fixative solution, and then immersed in 1% uranyl acetate for staining. Next, different concentrations of acetone were used for gradient dehydration. Finally, the embedded block was sectioned

and stained for visualization. Ultrastructural changes were observed by a transmission electron microscope.

2.6 | Measurement of serum levels of IgE and mucosal mast cell protease-1

The blood samples were collected 1 hr after the sixth OVA challenge according to the previous studies (Lee et al., 2018; O'Konek et al., 2020) and placed at room temperature for 30 min, and then, centrifuged to obtain serum. Enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA, USA) were used to measure serum levels of total IgE, OVA-specific IgE, and mucosal mast cell protease-1 (mMCP-1) following the manufacturer's instructions.

2.7 | Detecting the levels of cytokines

In order to determine cytokines, the blood, intestine, and spleen were removed from the mouse model 1 hr after the sixth OVA challenge (Lee et al., 2018; O'Konek et al., 2020), and then, the blood samples were centrifuged to obtain serum. The intestinal tract was first cut up with scissors, and then the tissue was homogenized and dissolved in a 1 ml lysis buffer containing protease inhibitor and phosphatase inhibitor, then centrifuged to obtain supernatant of tissue homogenates. In the meanwhile, the spleen tissue was homogenized to collect the cells. The spleen cells were cultured in cell medium in a humidified incubator at 37°C in the presence of 5% CO₂. Levels of cytokines (IL-4, IL-5, IL-13, IL-17A, and transforming growth factor- β (TGF- β)) were detected in the serum, supernatants of intestinal tissue homogenates, and spleen by ELISA kits according to the manufacturer's instructions.

2.8 | Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from small intestine with an RNeasy kit according to the manufacturer's instructions. The total RNA was reversely transcribed into cDNA by using oligo-dT primers. RT-qPCR was performed using LightCycler 480 system (Roche, Basel, Switzerland) and SYBR Green System (Roche, Basel, Switzerland). The PCR conditions were as follows: for 10 min at 95°C, followed by 45 cycles for 30 s at 95°C and for 30 s at 58–62°C. Glyceraldehyde

3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Primer sequences used for PCR are presented in Table 1.

2.9 | Western blot analysis

The intestinal tissue was cut into pieces and dissolved in RIPA lysis buffer containing protease inhibitors, centrifuged, and lysates were collected. The protein concentration was determined by a bicinchoninic acid (BCA) Assay Kit. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then, transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were sealed with 5% skim milk solution for 2 hr, and incubated with primary antibodies against GATA-3, ROR- γ t, Foxp-3, and GAPDH overnight at 4°C. Next, they were incubated with secondary antibodies at room temperature for 1 hr. The protein was detected by an enhanced chemiluminescent (ECL) kit (Thermo Fisher Scientific, Waltham, MA, USA), and the ImageJ software (National Institutes of Health, Bethesda, MD, USA) was employed for image processing.

2.10 | Flow cytometry analysis

First, CD4⁺T cells in the spleen were isolated using the CD4⁺T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Treg cells were stained with FITC-labeled anti-CD4, APC-labeled anti-CD25, and PE-labeled anti-Foxp3 antibodies. The cells were then fixed and permeabilized. After decanting supernatant, Fc receptors were blocked for 15 min, and cells were stained using PE-labeled anti-Foxp3 at 4°C for 30 min. FITC-labeled anti-mouse CD4 and PerCP-Cy5.5-labeled anti-IL17A were used to detect Th17 cells. CD4⁺T cells were stimulated for 4 hr, then, were washed, and surface-stained with FITC-labeled anti-CD4 antibody for 30 min at 4°C, and were resuspended. Cells were stained intracellularly with PerCP-Cy5.5-labeled anti-IL17A antibody for 30 min at 4°C. Data were acquired by the FC500 flow cytometer (Beckman Coulter Inc., Brea, CA, USA).

2.11 | Statistical analysis

Data were presented as mean $\pm$ SD. Comparisons were performed by using one-way analysis of variance, followed by the least significant difference test. Significant differences between two groups were

mRNA	Forward primer	Reverse primer
Mouse GAPDH	GAGTTGGGATAGGGCCTCTC	CAACTCCCACTCTTCCACCT
Mouse IL-17A	CTCTCAGGCTCCCTCTTCAG	ACTCTCCACCGCAATGAAGA
Mouse TGF- β	ACTGCTTCCCGAATGTCTGA	TCGCTTTGTACAACAGCACC
Mouse IL-4	TCTGCAGCTCCATGAGAACA	AACGAGGTCACAGGAGAAGG
Mouse IL-13	CTCTTGCTTGCCTTGGTGGTCT	TTGTGTGATGTTGCTCAGCTCC
Mouse IL-5	AGAGAAGTGTGGCGAGGAGA	CTCAGCCTCAGCCTTCCATTGC

TABLE 1 Primer sequences

compared by using the unpaired *t* test. $p < .05$ was considered statistically significant. All experiments were run in triplicate.

3 | RESULTS

3.1 | PNE alleviates the symptoms of allergy

As shown in Figure 1A, an OVA-induced food allergy model was established. As illustrated in Figure 1B,C, diarrhea occurred in all mice after the sixth provocation, and incidence of anaphylaxis was 83.3%. However, the incidences of diarrhea and anaphylaxis in PNE-50 group (diarrhea, 66.7%; anaphylaxis, 50%), PNE-100 group (diarrhea 50%; anaphylaxis, 16.7%), and PNE-200 group (diarrhea, 50%; anaphylaxis, 0%) were significantly reduced. As displayed in Figure 1D,E, OVA group showed the most severe symptoms of food allergy ($n = 6$; diarrhea, 2.83 ± 0.31 points; anaphylaxis, 2.50 ± 0.34 points). However, the PNE-based groups exhibited suppression of the OVA-induced diarrhea, as well as the incidence of anaphylaxis in all mice after the sixth provocation in PNE-50 group ($n = 6$; diarrhea, 1.67 ± 0.56 points; anaphylaxis, 1.00 ± 0.45 points), PNE-100 group ($n = 6$; diarrhea, 1.17 ± 0.54 points; anaphylaxis, 0.50 ± 0.34 points), and PNE-200 group ($n = 6$; diarrhea, 1.00 ± 0.45 points; anaphylaxis, 0.33 ± 0.21 points). In addition, compared with the control group, rectal temperature decreased by 8.48 ± 1.02 , 5.45 ± 1.12 , 3.17 ± 1.32 , and $2.75 \pm 1.08^\circ\text{C}$ in OVA, PNE-50, PNE-100, and PNE-200 groups, respectively (Figure 1F).

3.2 | PNE alleviates histopathological changes in intestine of mice

Under a light microscope, we observed that the number of neutrophils and other inflammatory cells was the highest in the OVA group.

Besides, PNE intervention was able to curb the accumulation of inflammatory cells (Figure 2A). Under an electron microscope, the villi of small intestine in OVA group were more disorganized and even shed than those in the control group. The villi were arranged orderly with only a small amount of shedding by the PNE pretreatment with different concentrations (50, 100, and 200 mg/kg) dose-dependently (Figure 2B).

3.3 | PNE reduces the levels of IgE and mMCP-1, and regulates response of T cells in the serum

We detected down-regulated levels of IgE and mMCP-1 in the serum that could be used to investigate the antiallergy capability of PNE. As depicted in Figure 3A, the expressions of total and OVA-specific IgE were higher in OVA group than those in control group, and those levels were remarkably reduced with PNE pretreatment at 50, 100, and 200 mg/kg dose-dependently. The expression of mMCP-1 in OVA group was higher than that in control group. However, compared with OVA group, mMCP-1 level in PNE-based groups was lower. Moreover, the repression effect of PNE at 100 mg/kg on mMCP-1 protein level was more potent than that of PNE at 200 mg/kg. We further detected the protein levels of IL-4, IL-5, and IL-13 in serum to evaluate the response of Th2 cells. The levels of IL-4, IL-5, IL-13 were higher in OVA group than those in control group, which were dose-dependently reduced in the PNE-based groups. Moreover, the inhibitory effects of PNE at 100 mg/kg on IL-5 and IL-13 protein expression were more potent than those of PNE at 200 mg/kg (Figure 3B). And we explored the effects of PNE on the Th17/Treg balance in serum. As displayed in Figure 3C, the food allergy in mice exhibited a significantly upregulated the protein level of Th17-associated cytokine (IL-17A), which was markedly reduced via PNE treatment at 50, 100, and 200 mg/kg dose-dependently in serum.

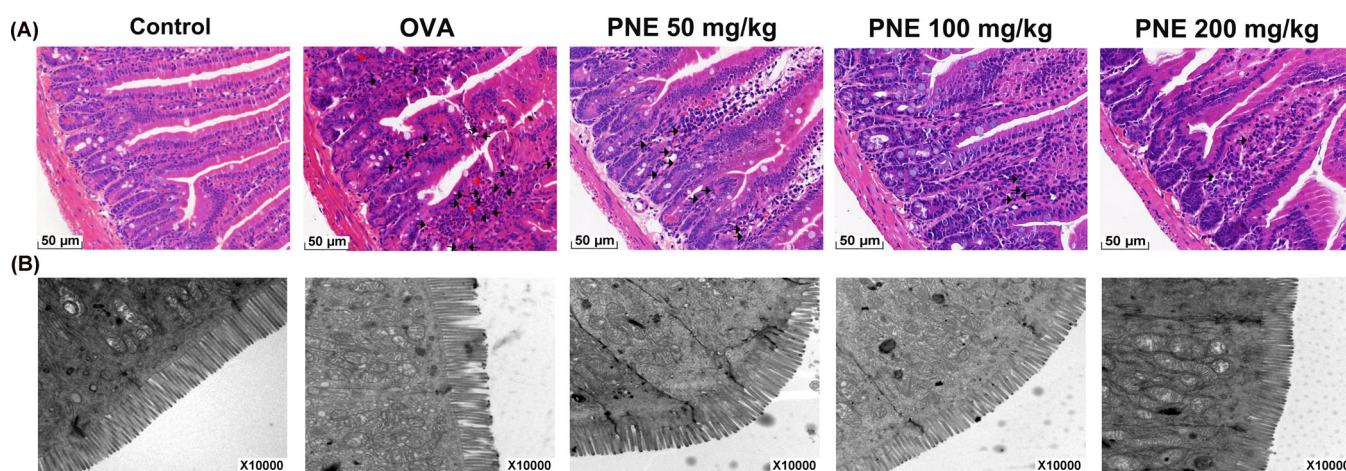


FIGURE 2 PNE alleviates intestine histopathological changes in mice. (A) The results of HE staining under a light microscope ($\times 400$). The black arrow is labeled with neutrophils, and the red arrow is labeled with eosinophils. Neutrophils and eosinophils were occasionally found in control group, and their number was increased in OVA group. PNE decreased the infiltration of the inflammatory cells. (B) The villi under an electron microscope ($\times 105$). The villi were arranged orderly and the structure was complete in the control group. The villi seriously damaged in OVA group. The villi were arranged orderly and slightly damaged in the PNE-based groups. All experiments were run in triplicate [Colour figure can be viewed at wileyonlinelibrary.com]

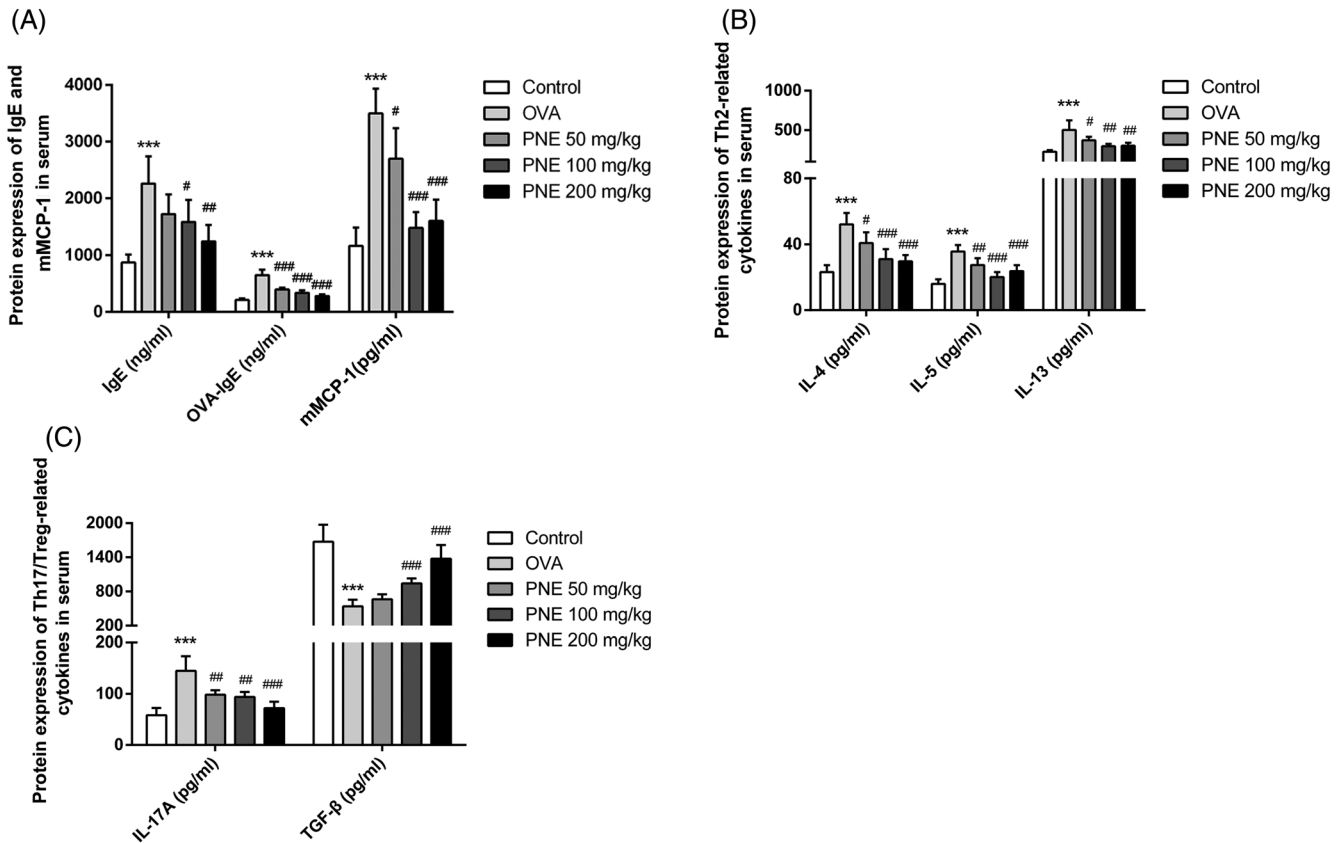


FIGURE 3 PNE inhibited the levels of IgE and mMCP-1, inhibits Th2 cell response, and regulates Th17/Treg response in serum. (A) Protein expression of IgE and mMCP-1 in serum. (B) Protein expression of IL-4, IL-5, IL-13 in serum. (C) Protein expression of IL-17A and TGF-β in serum. All experiments were run in triplicate. Data were presented as mean $\pm$ SD ($n = 6$). *** $p < .001$ compared with control group. # $p < .05$, ## $p < .01$, ### $p < .001$, compared with OVA group

Moreover, compared with control group, the level of Treg-associated cytokine (TGF-β) was down-regulated in the OVA group, which was up-regulated after PNE treatment dose-dependently in serum. However, there were no significant difference in protein level of TGF-β between PNE 50 group and OVA group.

3.4 | PNE inhibits Th2 response and regulates Th17/Treg response in the intestine

To understand the effect of PNE on Th2 cell response in the intestine, we investigated the changes of IL-4, IL-5, IL-13, and transcription factor GATA-3. IL-4, IL-5, IL-13, and GATA-3 mRNA levels in the intestine were increased in the OVA group, whereas PNE treatment significantly suppressed the expression of IL-4, IL-5, IL-13 and GATA-3 levels at 50, 100, 200 mg/kg concentration-dependently (Figure 4A). And PNE treatment also inhibited the expression of IL-4, IL-5, IL-13, and GATA-3 protein levels concentration-dependently. Moreover, there were no significant difference in level of IL-4 between PNE 50 group and OVA group (Figure 4C,E). The mRNA level of IL-17A, ROR-γt were down-regulated after PNE treatment dose-dependently compared with the OVA group. While the mRNA level of

TGF-β and Foxp-3 were up-regulated after oral exposure to PNE dose-dependently. In addition, the mRNA expression of IL-17A, ROR-γt, and TGF-β remained unaffected in PNE 50 group compared with OVA group (Figure 4B). PNE treatment also inhibited the expression of IL-17A and ROR-γt protein levels concentration-dependently. Furthermore, the expression of TGF-β and Foxp-3 protein levels were increased in PNE group compared with the OVA group (Figure 4D, F,G).

3.5 | PNE-mediate immunosuppression in the spleen of OVA-induced mice

Oral administration of PNE also down-regulated the expression of the Th2-related IL-4, IL-5, and IL-13 in the spleen dose-dependently compared with the OVA group (Figure 5A). Moreover, compared with control group, the protein level of Th17-related IL-17A was up-regulated in the OVA group, which was reduced after oral exposure to PNE. However, the expression of Treg-associated cytokine (TGF-β) was increased in PNE group compared with the OVA group concentration-dependently (Figure 5B). Whereas administrated PNE 50 mg/kg did not significantly alter IL-4 and TGF-β expression.

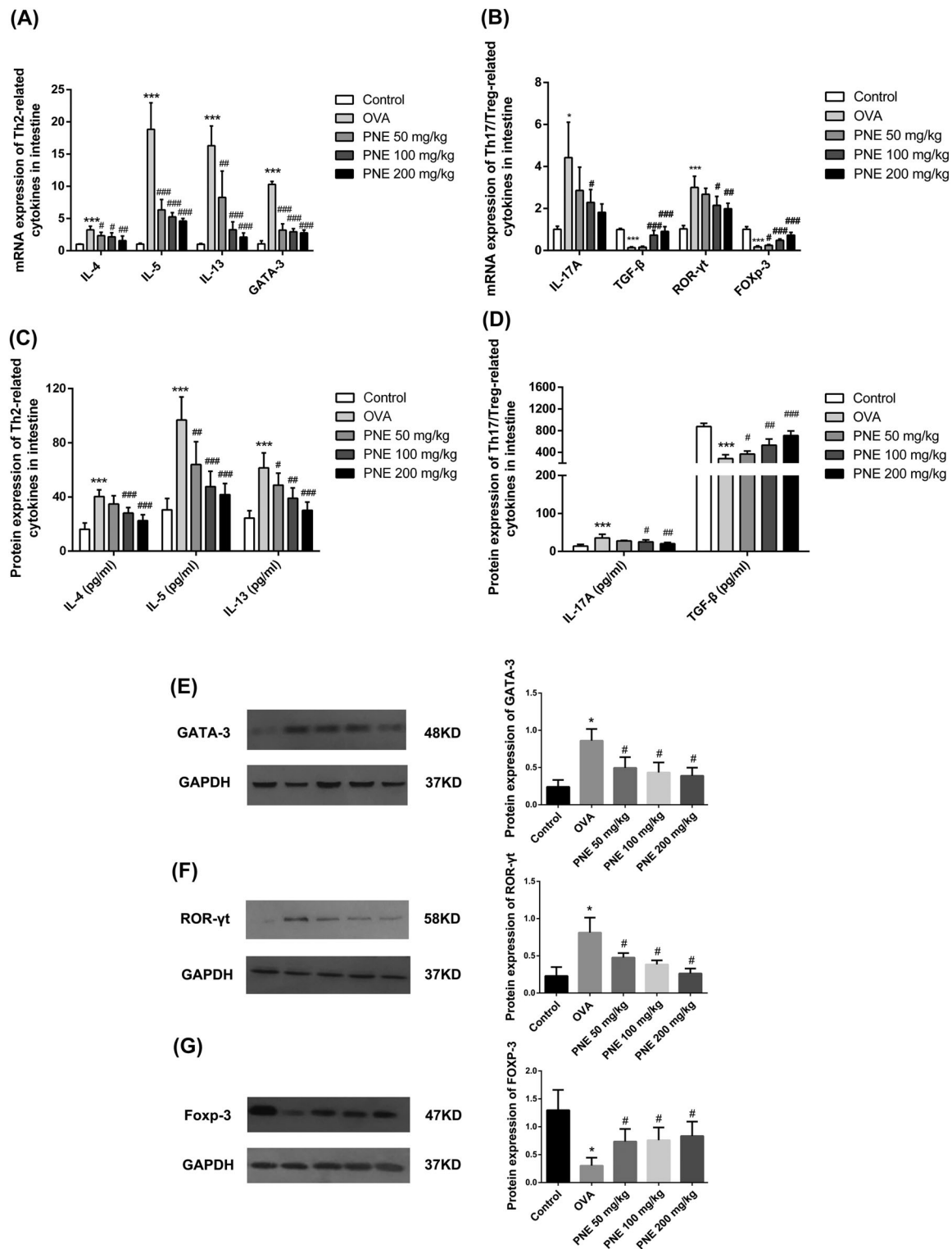


FIGURE 4 The expression of Th2, Th17, and Treg cells related cytokines in intestine. (A) mRNA expression of Th2-related IL-4, IL-5, IL-13, and GATA-3 in intestine (B) mRNA expression of Th17-related IL-17A and ROR-γt as well as Treg-related TGF-β and Foxp-3 in intestine. (C) Protein expression of IL-4, IL-5, IL-13 in intestine. (D) Protein expression of IL-17A and TGF-β in intestine. (E)–(G) showed protein expression of GATA-3, ROR-γt, and Foxp-3 in intestine. All experiments were run in triplicate. Data were presented as mean ± SD ($n = 6$). * $p < .05$, *** $p < .001$ compared with control group. # $p < .05$, ## $p < 0.01$, ### $p < .001$, compared with OVA group

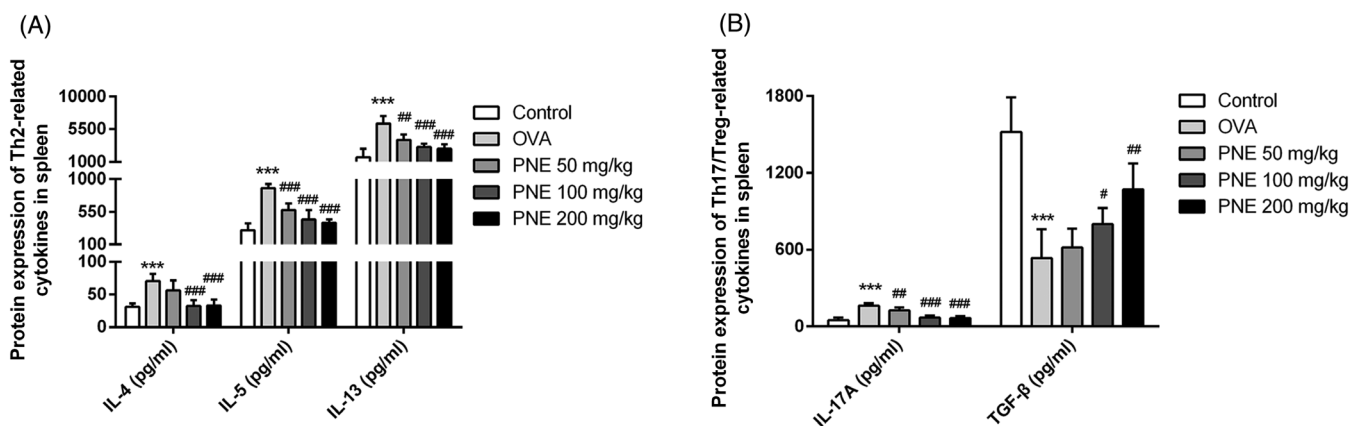


FIGURE 5 PNE inhibits Th2 cell response, and regulates Th17/Treg response in supernatant of splenocytes. (A) IL-4, IL-5, and IL-13 in supernatant of splenocytes. (B) Protein expression of IL-17A and TGF- β in supernatant of splenocytes. All experiments were run in triplicate. Data were presented as mean $\pm$ SD ($n = 6$). *** $p < .001$ compared with control group. # $p < .05$, ## $p < .01$, ### $p < .001$, compared with OVA group

3.6 | PNE regulates Th17/Treg ratio in OVA-induced allergy in mice

Flow cytometry was applied to assess the ratio of Th17/Treg cells in spleen. Remarkable elevations of Th17 cells in OVA group could be observed compared with control group, whereas the PNE group showed a notable reduction in Th17 cells dose-dependently (Figure 6A). Furthermore, decrease of Treg cells in OVA group could be observed compared with control group, whereas a notable increase in Treg cells was noted in the PNE group dose-dependently (Figure 6B). Additionally, the ratio of Th17/Treg cells in the PNE group was lower than that in OVA group (Figure 6C). The above-mentioned results suggested that PNE can inhibit Th17 response and promote Treg response to regulate Th17/Treg ratio.

4 | DISCUSSION

The present study showed that PNE may play a pivotal role in ameliorating allergic reactions via reducing IgE production, inhibiting degranulation of mast cells, decreasing Th2 cell response, and regulating the ratio of Th17/Treg. In this study, the antiallergic effect of PNE is obvious. After the administration of PNE, the allergy-related indicators (total IgE, etc.) and intestinal damage in mice with food allergy are ameliorated. In addition, the anaphylactic score and diarrhea score (representing the main clinical symptoms of food allergy) in the PNE group are significantly reduced.

Many requirements considered to be relevant in recent guidelines for best practice in natural products pharmacological research have been taken into account (Heinrich et al., 2020; Izzo et al., 2020). *Piper nigrum* is extensively applied because of its antioxidation, antiallergic, antiinflammatory, antidiarrhea, and gastric protection properties (Takoore et al., 2019). Bui et al. (2019) found that the allergic diseases were improved in BALB/c mice after oral administration of 50, 100, and 200 mg/kg PNE. Hritcu et al. (2015) found that oral administration of 50 or 100 mg/kg PNE had antianxiety effect on Alzheimer's disease

rats. And Agbor, Akinfiresoye, Sortino, Johnson and Vinson (2012) found that oral administration different doses of PNE (250 mg/kg and 1,000 mg/kg) can protect antioxidant status of atherogenic diet fed hamsters. We have done preliminary experiments (Figure S1) and set the concentration of 25, 50, 100, and 200 mg/kg, we found that the effect of 25 mg/kg was not very obvious. Therefore, we chose 50, 100, and 200 mg/kg to explore the effect of different doses of PNE. Based on the previous literature (Nair & Jacob, 2016), we have calculated the dose of the possible conversion in the human body. PNE 50, 100, 200 mg/kg were converted into human equivalent dose (HED) and the results were 4.05, 8.1, and 16.2 mg/kg in humans.

When an individual is exposed to allergens, the expressions of Th2-associated cytokines and IgE may be up-regulated, thereby mediating the expansions of mast cells, and inducing the infiltration of inflammatory cells in the intestine, which may increase the permeability of the intestine and lead to intestinal damage and anaphylaxis (Oyoshi, Oettgen, Chatila, Geha, & Bryce, 2014). To date, no definite treatment for food allergy has been proposed (Boyce, 2011). In the current research, we found that PNE (50, 100, and 200 mg/kg) ameliorated the symptoms of food allergy dose-dependently. Moreover, the administration of PNE dose-dependently inhibited OVA-induced accumulation of inflammatory cells in the small intestine, and alleviated damage to intestinal villi.

The mechanism of food allergy has not been fully elucidated. Previous studies indicated that inhibition of IgE production and degranulation of mast cells may improve allergic reactions (J. H. Kim et al., 2016; Zhang et al., 2019). When antigens cross-link with IgE and bind to Fc ϵ RI on the surface of mast cells, they may stimulate degranulation of mast cells, thereby releasing histamine, leukotriene, and other inflammatory mediators, which may cause symptoms of allergy (Albert et al., 2019). The present research detected the serum levels of IgE and mMCP-1, and it was revealed that administration of PNE (50, 100, and 200 mg/kg) dose-dependently inhibited the IgE production. Furthermore, administration of PNE at 100 mg/kg was more potent than PNE at 200 mg/kg to inhibit degranulation of mast cells.

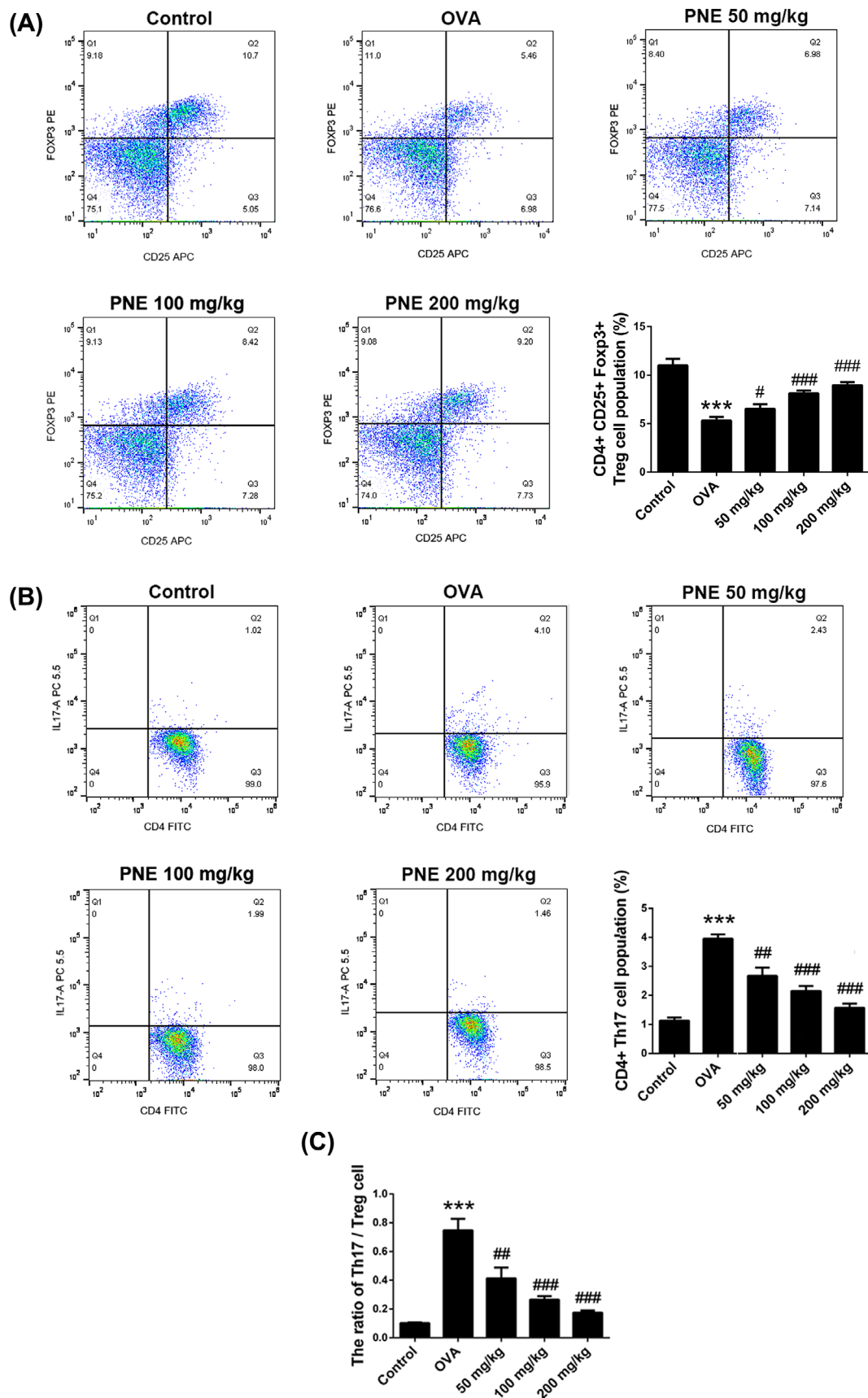


FIGURE 6 PNE administration reduces the number of Th17 and increase the number of Treg cells (A) Ratio of Th17/CD4+ T. (B) Ratio of Treg/CD4+ T. (C) PNE treatment down-regulates the ratio of Th17/Treg cells in the spleen of mice. All experiments were run in triplicate. Data were presented as mean $\pm$ SD ($n = 3$). * $p < .05$, compared with the control group. # $p < .05$, ## $p < .01$, ### $p < .001$, compared with OVA group [Colour figure can be viewed at wileyonlinelibrary.com]

A study reported that allergic diseases are mainly accompanied by the increased levels of Th2-associated cytokines (IL-4, IL-5, IL-13, and GATA-3), which can mediate dysregulation and abnormal immune responses (Noval-Rivas & Chatila, 2016). Dendritic cells (DCs) can capture food allergens and present them to naïve T cells, which may induce differentiation of Th2 cells. Up-regulated levels of Th2-associated cytokines and IgE may mediate the degranulation of mast cells and increase the permeability of intestine, causing symptoms of food allergy (Oyoshi et al., 2014). Lee et al. (2018) pointed out that suppressed Th2 reaction can effectively alleviate food allergy. Kim and Lee (2009) showed that administration of PNE can ameliorate airway inflammation by regulating response of Th cells to inflammation. However, in the process of food allergy, the relationship between PNE and Th2 cell-associated cytokines is less clear. We studied the effects of PNE on response of Th2 cells to inflammation, and it was shown that the protein and mRNA levels of IL-4, IL-5, IL-13, and GATA-3 were elevated by the allergy induction, while the administration of PNE inhibited the expressions in serum, intestine and spleen at 50, 100, 200 mg/kg dose-dependently. Moreover, inhibition of PNE at 100 mg/kg on protein expression of IL-5 and IL-13 in serum was more potent than that of PNE at 200 mg/kg. These results above indicated that PNE may improve the food allergy by effectively inhibiting Th2 response.

Dysfunction of Treg cells can lead to allergic diseases, while inducing Treg cells can prevent food allergy development by inhibiting Th17 cells (Wang et al., 2012). Our study assessed the effects of PNE on Th17 and Treg cells in food allergy, in addition to its possible mechanisms. Our findings disclosed that compared with control group, the number of Th17 cells was significantly elevated, while the number of Treg cells were reduced in the OVA group. After PNE treatment, the imbalance ratio of Th17/Treg cells could be ameliorated, and the inhibitory effect was dose-dependent.

We further investigated the mechanism of improving the Th17/Treg balance by administration of PNE. Previous researches proved that TGF- β can inhibit Th2 and Th17 cells in allergic diseases, inducing Foxp-3 production to promote the naïve T cells into Treg cells, in addition to suppression of IgE production, thereby improving food allergy (Bacchetta, Gambineri, & Roncarolo, 2007; Chen et al., 2003). A recent study found that ROR- γ t can promote the differentiation of Th17 cells (Ivanov et al., 2006). In the present study, in OVA group, the expressions of TGF- β and Foxp-3 were reduced, while those of ROR- γ t and IL-17A were elevated compared with those in control group in serum, intestine, and spleen. However, the levels of TGF- β and Foxp-3 in the PNE-based groups were up-regulated compared with those in the OVA group, while the expressions of ROR- γ t and IL-17A were down-regulated. These results suggested that PNE may dose-dependently ameliorate the Th17/Treg balance by influencing the levels of TGF- β and ROR- γ t.

5 | CONCLUSIONS

Our research elucidated that PNE plays a positive role in attenuating the allergic response by reducing the production of IgE and

degranulation of mast cells, inhibiting the infiltration of inflammatory cells, improving the histopathology of the intestine, and decreasing Th2 cell response. Moreover, PNE may dose-dependently ameliorate the Th17/Treg balance by modulating the expressions of TGF- β and ROR- γ t, thereby attenuating OVA-induced symptoms of food allergy. In summary, our results indicated the efficacy and potential mechanism of PNE as an antiallergic drug to treat food allergy.

6 | LIMITATION

At present, there is no definitive and effective treatment for food allergy, thus our research failed to provide the corresponding positive control group. Moreover, this study focused on the preventive effect of PNE on food allergy and the therapeutic effect of PNE on food allergy needs further research. In addition, the 50–1,000 mg/kg of PNE in previous literature studies did not report toxicological reactions (Agbor et al., 2012; Hritcu et al., 2015), however, there is no report on the threshold of toxicological concern of PNE that no specific toxicological data can be provided. These are the limitation of this study.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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